Responses of glutamine transport in cultured rat skeletal muscle to osmotically induced changes in cell volume

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1. In order to investigate the relationship between cellular hydration state and the rate of glutamine transport, tracer glutamine uptake into primary rat myotubes was studied at external osmolalities of 170, 320 or 430 mosmol kg^{-1}.

2. Incubation of myotubes with glutamine (2 mM; 30 min) at 320 mosmol kg^{-1} increased cell volume and glutamine transport (by 35 and 36%, respectively); insulin (66 nM; 30 min) also increased cell volume and glutamine transport (by 22 and 40%, respectively) and the effects of insulin and glutamine combined were additive. The increase in glutamine uptake following glutamine pre-incubation represented an increase in V_{max} of Na^+-dependent glutamine transport.

3. There was an inverse relationship between myotube glutamine transport and external osmolality after 30 min exposure.

4. During hyposmotic (170 mosmol kg^{-1}) exposure there were large, rapid increases of cell volume and glutamine transport; the latter increased transiently (during the cell swelling phase) by a maximum of ~80% at 2 min, (due to an increased V_{max} for Na^+-dependent glutamine transport) then decayed to a new elevated steady state after 30 min exposure.

5. During hyperosmotic (430 mosmol kg^{-1}) exposure there were rapid decreases in glutamine transport and myotube cell volume (both by ~30%) to values which were maintained for at least 15 min.

6. The volume-sensitive glutamine transport process features characteristics of the insulin-sensitive system N^m transporter.

7. Modulation of Na^+-dependent glutamine transport by insulin and cell volume changes may contribute towards regulation of muscle metabolism.

Glutamine, the major substrate of the insulin-sensitive, Na^+-dependent transport system N^m in the sarcolemma, has anabolic effects on carbohydrate and protein metabolism in rat and human skeletal muscle (Rennie & Scislowski, 1989; Scislowski, Niblock, Lindsay, Weryk, Watt & Rennie, 1989; Stehle, Zander, Albers, Puchstein, Lawin & Fürst, 1989; Barua, Wilson, Downie, Weryk, Cuschieri & Rennie, 1992; Varnier, Leese & Rennie, 1995). System N^m has the highest capacity of any insulin-sensitive amino acid transporter in skeletal muscle (Hundal, Rennie & Watt, 1987, 1989; Ahmed, Taylor & Rennie, 1990) and insulin stimulation of Na^+-coupled glutamine uptake is likely to result in osmotic cell swelling of skeletal muscle. Cell swelling and subsequent volume regulation during concentrative uptake of amino acids (including glutamine), as occurs in the absorptive state, are now recognized as likely signals modulating the metabolic state of the liver (Häussinger, Lang, Bauers & Gerok, 1990; Häussinger, Lang & Gerok, 1994). Furthermore, recent studies indicate that cell swelling may be an anabolic signal involved in effecting the hepatic response to insulin (Vom Dahl, Hallbrucker, Lang & Häussinger, 1991) and that changes of less than 10% of initial volume may nevertheless be important (Häussinger *et al.* 1990, 1994; Bode & Kilberg, 1991; Vom Dahl *et al.* 1991).

Hepatic system N (which has functional similarities to system N^m) is activated in response to swelling of liver cells (Bode & Kilberg, 1991). The likelihood that this also occurs with system N^m opens up the possibility of sophisticated feedback loops for metabolic control involving glutamine; indeed, we hypothesize that modulation of glutamine transport may be an integral component of metabolic control systems in skeletal muscle. Cell swelling has been hypothesized to be a part of the anabolic response to feeding in skeletal muscle (Parry-Billings, Bevan, Opara & Newsholme, 1991; Häussinger, Roth, Lang & Gerok, 1993) but direct evidence for system N^m activation by muscle cell swelling is lacking at present, although net glutamine efflux from incubated rat skeletal muscle does decrease after cell swelling of a magnitude (5–10%, Parry-Billings *et al.* 1991)

similar to that having metabolic effects in liver cells (Häussinger *et al.* 1990, 1994; Vom Dahl *et al.* 1991). We have shown previously that rat skeletal muscle in primary culture provides a useful system for studying amino acid transport and metabolism in skeletal muscle (Tadros, Taylor & Rennie, 1993*a*; Tadros, Willhoft, Taylor & Rennie, 1993*b*; Low, Rennie & Taylor, 1994). We have, therefore, used this preparation to investigate the relationship between glutamine transport activity, muscle cell volume and insulin action.

METHODS

Cell culture

Skeletal muscle cells were harvested from the thigh muscles of 1-day-old neonatal rats (killed by cervical dislocation); dissected limbs were minced, washed and disaggregated using 0·25% trypsin as previously described (Tadros *et al.* 1993*a*; Low *et al.* 1994). Cells were cultured on collagen-coated, 24-well plates (Becton & Dickinson, Cowley, UK) in growth medium (25% medium 199; 65% Dulbecco's modified Eagle's medium; 10% fetal calf serum) supplemented by 2 mM glutamine at 37 °C in 5%CO_2–95% air. Myogenesis was promoted by replacement of calf serum by 10% horse serum and 3% chick embryo extract on day 5 of culture as described previously (Tadros *et al.* 1993*a*; Low *et al.* 1994). All cell culture media and sera were obtained from Life Technologies (Gibco BRL, Paisley, UK) except medium 199 which was obtained from Sigma.

Transport studies

All experiments were performed on 10-day-old, confluent, multinucleated myotubes. Uptake of L-[G-^{3}H]glutamine, L-[^{3}H]glutamate, L-[^{3}H]glucose or [^{14}C]methylaminoisobutyrate (MeAIB) (all at 0·05 mM unless stated otherwise) into myotubes was measured over one minute (initial rate) in NaCl, LiCl or choline chloride (ChoCl) buffer with D-[^{14}C]mannitol (or [^{3}H]mannitol for [^{14}C]MeAIB studies) as extracellular markers with the ^{3}H: ^{14}C ratio kept at 5:1 (Tadros *et al.* 1993*a*; Low *et al.* 1994). Uptake was stopped by rapidly removing the uptake buffer by aspiration and washing the cells with ice cold saline. For liquid scintillation spectrometric assay of radioactivity and protein determination (by the bicinchoninic acid method (Smith *et al.* 1985)), cells were first solubilized in 0·5 M NaOH. All radiotracers were obtained from NEN Research Products (Stevenage, UK) except glutamine which was obtained from Amersham International (Aylesbury, UK). Insulin (100 U ml^{-1}) was obtained from Novo Nordisk (Crawley, UK). 5-Nitro-2-(3-phenyl propyl amino)-benzoate (NPPB) was obtained from Calbiochem Novabiochem (Nottingham, UK). All other chemicals were obtained from Sigma.

Measurement of cell volume (cell hydration)

Cell hydration was estimated from the ^3H_2O space (9·5 kBq per well) using D-[^{14}C]mannitol (1·9 kBq per well) as the extracellular marker. At the end of each measurement period, as dictated by the experiments, the bulk of the radioactive supernatant was removed by aspiration but, to minimize loss of the rapidly exchanging ^3H_2O tracer, the cells were not washed prior to solubilization and radioactivity assay. Preliminary studies demonstrated that ^3H_2O equilibrated with myotube water within 2 min (Fig. 1); thereafter, this period was used for measurements of cell volume. This preliminary data also showed that the effective 'extracellular space' of myotube monolayers estimated using [^{14}C]mannitol was: (a) independent of incubation period (Fig. 1); (b) of similar magnitude to that estimated using [^{14}C]sucrose (data not shown); and (c) unaffected by experimental manoeuvres which altered cell volume (30 min pre-incubation in medium of osmolality 170 or 430 mosmol kg^{-1}; data not shown); these data confirm the utility of tracer mannitol as an extracellular marker for skeletal muscle preparations (Hundal *et al.* 1987).

Experimental procedures

The basic experimental medium contained (mM): 121 NaCl, 4·9 KCl, 2·5 $MgSO_4$, 20 tris(hydroxymethyl)aminomethane hydrochloride and 1 $CaCl_2$. Glutamine (2 mM) and/or insulin (66 nM) were added to this experimental medium where indicated. The osmolality of all experimental media was measured using a Clandon Gonotec Osmomat 030 osmometer (Gonotec, Berlin, Germany). The osmolality of the normosmotic experimental medium was adjusted, with sucrose, to match the osmolality of each particular batch of culture media (320 ± 10 mosmol kg^{-1}).

In experiments involving pre-incubation with glutamine and/or insulin followed by glutamine uptake, the pre-incubation solution was rapidly removed by aspiration and the cells were washed carefully with 2 × 1 ml of uptake buffer before measurement of glutamine uptake as described above. Insulin was used at 66 nM because this concentration was previously found (Tadros *et al.* 1993*a*) to stimulate maximally myotube glutamine uptake.

In all experiments involving altered extracellular osmolality, myotubes were exposed for up to 60 min to a modified medium containing 60 mM NaCl with sucrose added to achieve the osmolality (170–500 mosmol kg^{-1}) dictated by the experimental designs; measurements of 0·05 mM L-[^{3}H]glutamine transport or ^3H_2O space were made over the final one or two minutes of this period, respectively. In preliminary experiments it was shown that these manoeuvres did not produce irreversible adverse effects on myotubes.

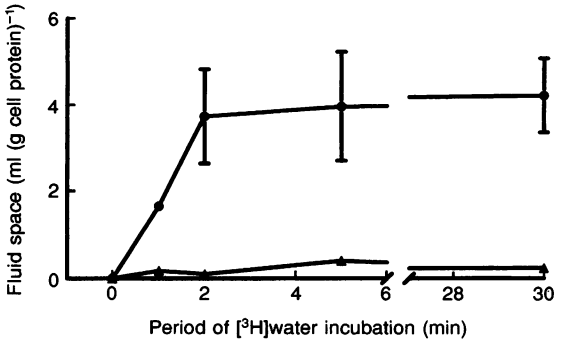

Figure 1. Time course of cell ^3H_2O uptake and equilibration in skeletal muscle myotubes

The quantity of ^3H_2O accumulated by myotubes (shown as the ^3H_2O space; ●) and the effective 'extracellular space' ([^{14}C]mannitol space; ▲) were measured over the denoted incubation periods in ^3H_2O/[^{14}C]mannitol-labelled NaCl medium. Values are means ± S.E.M. for three preparations.

Table 1. Effects of glutamine and insulin on glutamine transport activity and cell volume of skeletal muscle myotubes

Condition	Glutamine transport (pmol min^{-1} (mg protein)$^{-1}$)	Cell hydration (ml water (g protein)$^{-1}$)
Control	327 ± 58	2·95 ± 0·33
+Insulin	459 ± 71 **	3·60 ± 0·50 *
+Glutamine	435 ± 73 **	4·08 ± 0·73 *
+Gln +Insulin	605 ± 140 **	5·40 ± 1·08 *

[^{3}H]-labelled glutamine (0·05 mM) transport and cell volume (^3H_2O space) were measured over 1 or 2 min, respectively, in NaCl medium after 30 min exposure to basic experimental medium without or with 2 mM glutamine (Gln) and without or with 66 nM insulin. Values are means ± S.E.M. of 4 preparations. Statistical significance was determined using the paired t test: * $P < 0·05$ and ** $P < 0·01$.

Data analysis and presentation

Data are presented as mean values ± S.E.M. for n myotube preparations; each experimental measurement in an individual preparation was performed in triplicate using three separate wells in a culture plate. Statistical significance was determined using either the paired t test or Student's t test as indicated. Correlation and regression analyses were performed using commercial software (Slide-Write Plus, Advanced Graphics Software Inc., Carlsbad, USA) where indicated.

RESULTS

Myotubes incubated for 30 min in medium (121 mM NaCl) containing glutamine (2 mM) or insulin (66 nM) had higher cell volumes (35 ± 12 and 22 ± 7% increases in cell hydration, respectively,) and glutamine transport activity (36 ± 12 and 40 ± 6% increases, respectively) than controls incubated in the absence of glutamine and insulin, see Table 1). The effects of glutamine and insulin combined were additive (Table 1); indeed, they appeared to be marginally (~10%) higher than the sum of the effects produced by glutamine or insulin alone. Experiments involving pre-incubation with insulin or glutamine were also performed in Dulbecco's modified Eagle's medium which contains physiological concentrations of amino acids and glucose. The results obtained from these experiments were similar to those shown in Table 1 (data not shown).

We next examined the ion and concentration dependence of the glutamine-induced increase in transport. Glutamine uptake (at 0·05, 0·2, 0·5 and 1 mM) in 121 mM NaCl or ChoCl uptake medium was measured in myotubes which had been pre-incubated for 30 min in medium (121 mM NaCl or ChoCl) without or with 2 mM glutamine. Uptake of glutamine in ChoCl medium was unaffected by pre-incubation of the cells in medium containing 2 mM glutamine at all concentrations examined (data not shown). However, there was a significant increase in Na^+-dependent glutamine uptake in cells which had been pre-incubated with 2 mM glutamine (Fig. 2*A*), apparently due to an elevated V_{max} for glutamine transport (Fig. 2*B*). We have shown previously (Tadros *et al.* 1993*a*) that insulin

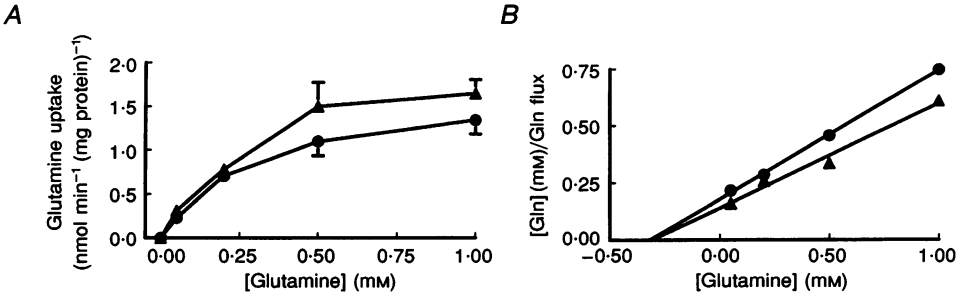

Figure 2. Effect of 30 min exposure of myotubes to 2 mM glutamine (Gln) on glutamine uptake at different external glutamine concentrations

A, Na^+-dependent glutamine uptake. [^{3}H]-Labelled glutamine (0·05, 0·2, 0·5 and 1 mM) transport was measured over one minute in 121 mM NaCl and 121 mM ChoCl medium following a 30 min incubation without (●) or with (▲) 2 mM glutamine. The uptake of glutamine in ChoCl medium was deducted from that in NaCl to give Na^+-dependent glutamine uptake. Values are means ± S.E.M. for 6 preparations. *B*, using a Hanes plot, the mean values for Na^+-dependent glutamine uptake from *A* were used to estimate the K_m and V_{max} for glutamine transport without (●) or with (▲) 2 mM glutamine. The slope of the line represents $1/V_{max}$ and the intercept on the x axis gives K_m. Transport V_{max} in cells pretreated with glutamine (2·19 ± 0·21 nmol min^{-1} (mg protein)$^{-1}$) was significantly higher ($n = 6$; $P < 0·05$) than in controls (1·77 ± 0·04 nmol min^{-1} (mg protein)$^{-1}$) whereas the respective K_m values (~310 μM) were similar to one another.

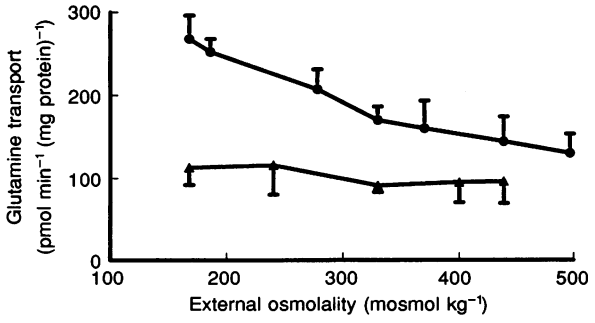

Figure 3. Effects of 30 min exposure of myotubes to different osmolalities on glutamine transport

[^{3}H]-labelled glutamine (0·05 mM) transport was measured over 1 min at the end of a 30 min exposure of myotubes to media with the osmolalities indicated. NaCl (●) or ChoCl (▲) concentration was 60 mM in all experiments: osmolality was adjusted using sucrose. Values are mean ± S.E.M. of 3 preparations.

stimulated Na^+-dependent glutamine transport by increasing its V_{max}.

Having established an association between myotube cell volume and glutamine transport activity, we investigated the effect of experimental changes in cell volume (induced by alterations of external osmolality from a nominal control value of 320 mosmol kg^{-1}) on myotube glutamine transport. In these, and all subsequent experiments, the external NaCl concentration was reduced to 60 mM in order to accommodate the required changes in osmolality. This change resulted in reduced 'control' rates of glutamine uptake (i.e. those rates measured at 320 mosmol kg^{-1} osmolality) compared with the data shown in Table 1. This effect on glutamine uptake also occurred if external Na^+ was reduced to 60 mM independently of Cl^- (i.e. glutamine uptake decreases from 324·8 ± 40·5 pmol min^{-1} (mg protein)$^{-1}$ at 121 mM NaCl to 221 ± 22·7 pmol min^{-1} (mg protein)$^{-1}$ at 60 mM NaCl plus 61 mM ChoCl), indicating that the reduced glutamine transport was related primarily to the lowering of external Na^+ rather than, for example, Cl^--related effects on cell-membrane potential. Preliminary experiments showed that myotubes tolerated exposure to media of between 170 and 500 mosmol kg^{-1} for up to one hour; beyond these limits myotubes became dissociated from the culture wells and/or lysed. We used a standard 30 min exposure period in a series of experiments which showed there was an inverse relationship between myotube glutamine transport and external osmolality (Fig. 3). The predominant effect of osmolality change was on a Na^+-dependent component of glutamine transport (Fig. 3).

We next examined the time course of changes in glutamine transport and cell volume of myotubes during hyposmotic (170) or hyperosmotic (430 mosmol kg^{-1}) exposure. During hyposmotic exposure (Fig. 4*A*) there was a rapid but transient increase (reaching a maximum of ~80% after 2 min) of inward glutamine transport. This initial increase in transport appeared to decay with time but remained higher than control levels over a 30 min exposure period. Following hyperosmotic exposure there was a rapid decrease in glutamine transport (Fig. 4*A*; by ~30%), which appeared to be sustained over 30 min. Myotube cell volume showed rapid changes after anisosmotic transfer, as expected (Fig. 4*B*). There appeared to be some return towards initial cell volume within the 30 min experimental

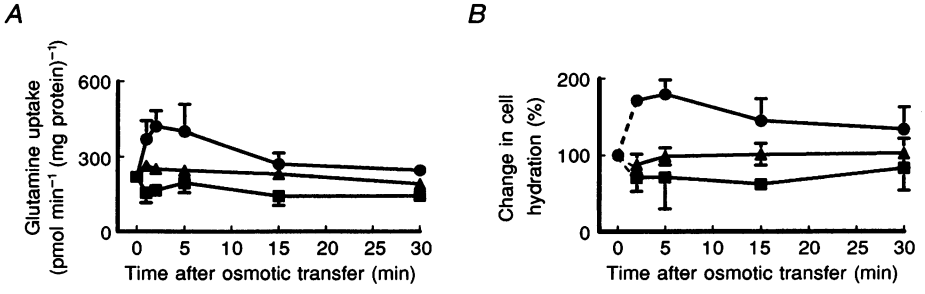

Figure 4. Time course of effects of exposure to hyperosmotic (430) and hyposmotic (170 mosmol kg^{-1}) conditions on skeletal muscle myotubes

A, muscle glutamine uptake. [^{3}H]-labelled glutamine (0·05 mM) transport was measured over the final one minute of timed periods of exposure of myotubes to media with osmolalities of 170 (●); 320 (▲) and 430 (■) mosmol kg^{-1}. NaCl concentration was 60 mM in all experiments; osmolality was adjusted using sucrose. Values are means ± S.E.M. of 4–6 preparations. *B*, muscle cell volume. Myotube cell volume (^3H_2O space) was measured over the final two minutes of timed periods of exposure of myotubes to media with osmolalities as *A*. NaCl concentration was 60 mM in all experiments; osmolality was adjusted using sucrose. Values are means ± S.E.M. of 4–5 preparations.

Table 2. Myotube amino acid and glucose uptake during hyposmotic exposure

Condition	0·05 mM solute uptake (pmol min^{-1} (mg protein)$^{-1}$)		
	at 320 mosmol kg^{-1}	at 170 mosmol kg^{-1}	
NaCl + [^{3}H]glutamine	169 ± 27	313 ± 38**	(10)
NaCl + [^{3}H]glutamine + MeAIB	182 ± 17	373 ± 52*	(3)
LiCl + [^{3}H]glutamine	196 ± 43	343 ± 57*	(5)
ChoCl + [^{3}H]glutamine	80 ± 13	90 ± 41	(3)
NaCl + [^{3}H]glucose	262 ± 69	220 ± 49	(3)
NaCl + [^{3}H]glutamate	293 ± 87	226 ± 50	(5)
NaCl + [^{14}C]MeAIB	22·2 ± 4·1	9·2 ± 2·7*	(3)

Uptake of 0·05 mM radiolabelled amino acid or glucose into myotubes was measured over 1 min after a 2 min exposure to the indicated osmolalities. NaCl, LiCl or ChoCl concentrations were 60 mM throughout: osmolality was adjusted using sucrose. Unlabelled MeAIB was added at 5 mM where indicated. Values are means ± S.E.M. from (n) preparations. Statistical significance of the difference between uptake at the two osmolalities was assessed using the paired t test: * $P < 0·05$, ** $P < 0·01$.

period, notably after hyposmotic exposure, probably reflecting cell volume regulatory responses of the cell.

Glutamine uptake (0·05–1 mM) was measured in hyposmotic (170) and isosmotic (320 mosmol kg^{-1}) medium with or without sodium ions. There was no change in Na^+-independent glutamine uptake (measured in medium containing 60 mM ChoCl) following exposure to hyposmotic media (data not shown) but there was an increase in Na^+-dependent glutamine uptake at all glutamine concentrations after the same experimental treatment (Fig. 5*A*). This increase in Na^+-dependent glutamine uptake represented an increase in the V_{max} for glutamine transport (Fig. 5*B*) without a change in transport K_m.

The increased glutamine uptake seen after 2 min of hyposmotic exposure was not inhibited by the system A substrate MeAIB (Table 2) nor was it observed when external Na^+ was replaced by Cho^+, but Li^+ was accepted as a substitute for Na^+. Uptake of L-glutamate (which is not a good substrate for the glutamine transporter in skeletal muscle (Hundal *et al.* 1989)) and D-glucose by myotubes were unaffected by short-term (2 min) hyposmotic exposure (Table 2) whereas MeAIB uptake was decreased. The effect of the anion channel blockers, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS; 0·2 mM), 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; 0·2 mM) and NPPB (0·1 mM) on the induced uptake of glutamine were also evaluated. The increase observed in

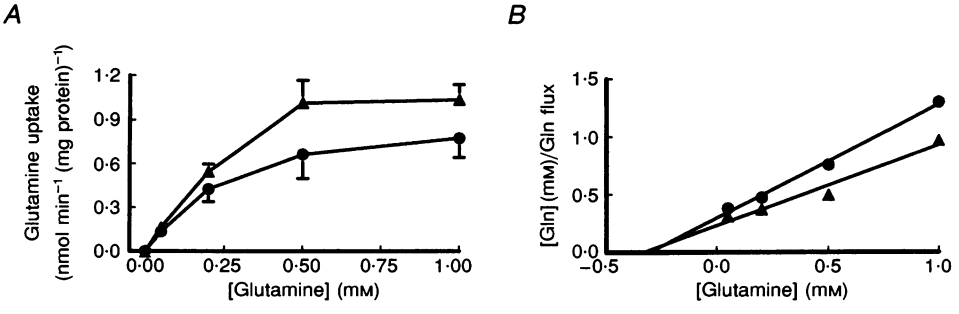

Figure 5. Effect of 2 min hyposmotic exposure in myotubes on glutamine (Gln) uptake at various external glutamine concentrations

A, Na^+-dependent glutamine uptake. [^{3}H]-labelled glutamine (0·05, 0·2, 0·5 and 1 mM) transport was measured over the final minute of a 2 min exposure to 170 (▲) or 320 (●) mosmol kg^{-1} medium containing 60 mM NaCl or ChoCl. The uptake of glutamine in ChoCl medium was deducted from that in NaCl to give Na^+-dependent glutamine uptake. Values are means ± S.E.M. for six preparations. *B*, using a Hanes plot, the mean values for Na^+-dependent glutamine uptake from *A* were used to determine the K_m and V_{max} for glutamine uptake in 320 (●) and 170 (▲) mosmol kg^{-1}. The slope of the line represents $1/V_{max}$ and the intercept on the x axis gives K_m. Transport V_{max} increased from 1·02 ± 0·021 to 1·46 ± 0·21 pmol min^{-1} (mg protein)$^{-1}$ ($n = 6$; $P < 0·05$) after exposure to hyposmotic media; with no appreciable change in K_m (327 and 309 μM in hyposmotic and isosmotic conditions, respectively).

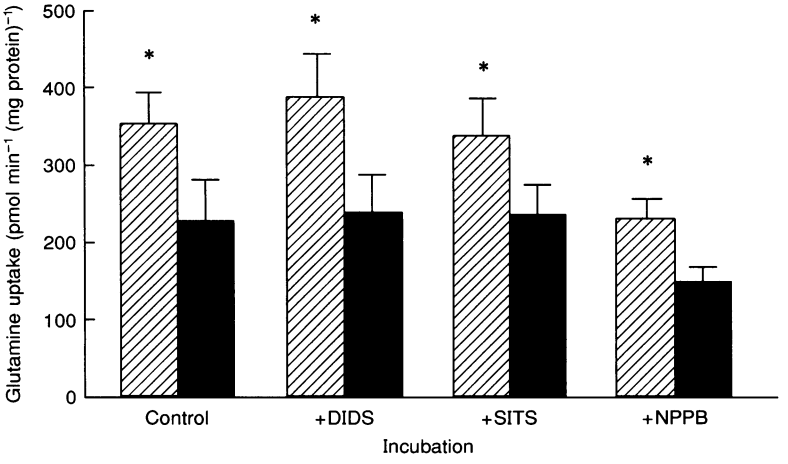

Figure 6. Effect of 2 min of hyposmotic exposure on glutamine uptake in myotubes in the presence of DIDS, SITS and NPPB

[^{3}H]-labelled glutamine (0·05 mM) transport was measured over the final one minute of a two minute exposure of myotubes to media with the osmolalities of 170 (▨) and 320 (■) mosmol kg^{-1}. DIDS (0·2 mM), SITS (0·2 mM) and NPPB (0·1 mM) were added for 2 min (i.e. at the same time as the hyposmotic exposure began). NaCl concentration was 60 mM in all experiments, osmolality was adjusted using sucrose. Values are means ± S.E.M. of 4–5 preparations. Statistical significance of the difference between uptake at the two osmolalities was assessed using the paired t test: * $P < 0·05$.

myotube glutamine uptake following exposure to hyposmotic media was unaffected by the presence of SITS or DIDS (Fig. 6). Basal glutamine uptake decreased in the presence of NPPB but the stimulation of glutamine uptake after hyposmotic exposure was still observed (Fig. 6).

DISCUSSION

The present results demonstrate that the effects of insulin on myotubes include short-term cell swelling associated with increased glutamine transport. The insulin-sensitive glutamine transporter in myotubes is the Na^+-dependent system N^m (Tadros *et al.* 1993*a*). The presence of extracellular glutamine augments both these insulin-stimulated effects: thus the anabolic effects of insulin and cell swelling in skeletal muscle may be enhanced by associated increases in glutamine uptake, providing a partial explanation for some of the anabolic effects of glutamine in skeletal muscle (MacLennan, Brown & Rennie, 1987; MacLennan, Smith, Weryk, Watt & Rennie, 1988; Rennie & Scislowski, 1989; Stehle *et al.* 1989; Varnier *et al.* 1995). Furthermore, we found muscle cell shrinkage (as has been reported to occur in a variety of circumstances of disease or injury (Häussinger *et al.* 1993)) to be associated with a reduction in the rate of glutamine uptake; such an effect may account in part for the lowered cell glutamine concentrations and increased net efflux of muscle glutamine characteristic of many catabolic states (Rennie *et al.* 1986; Häussinger *et al.* 1993).

Our group has demonstrated a positive correlation between extracellular (and intramuscular) glutamine concentration and the rate of rat and human muscle protein synthesis (Rennie *et al.* 1986; MacLennan *et al.* 1987). This anabolic effect of glutamine was most marked in the presence of insulin. Glutamine also stimulates muscle glycogen synthesis in rat and human muscle (Scislowski *et al.* 1989; Varnier *et al.* 1995) and inhibits muscle protein breakdown (MacLennan *et al.* 1988). One possible mechanism for triggering these anabolic effects might involve increased Na^+-dependent glutamine uptake and the resultant, osmotically induced, cell swelling. Glutamine has the highest plasma and muscle cell concentration of any amino acid and both the basal- and insulin-stimulated capacities for glutamine transport into muscle are greater than those of other amino acids at physiological concentrations (Hundal *et al.* 1987, 1989). Glutamine is, therefore, likely to exert the greatest effects of any natural amino acid on muscle cell volume and osmolality. The physiological range of plasma glutamine concentrations is of the order 0·5–1·5 mM (0·2–2 mM in certain diseases), depending upon nutritional status (Abumrad *et al.* 1989). The range of our experimental manipulations is, therefore, only marginally out with that found *in vivo* and, although our abrupt 0–2 mM glutamine changes are supraphysiological challenges, it is likely that physiological and pathophysiological changes in glutamine availability (e.g. after a meal or injury) will also be capable of significantly affecting muscle cell volume. We have estimated the potential magnitude of an osmotically induced increase in myotube volume following 30 min exposure to 2 mM glutamine, assuming a net transport stoichiometry of 1 glutamine : 1 Na^+ + 1 Cl^- to maintain electroneutrality and a net glutamine uptake rate at 2 mM of 2 nmol min^{-1}

(mg protein)$^{-1}$ (i.e. mid way between V_{max} values with or without glutamine as shown in the legend to Fig. 2). Such an accumulation of glutamine plus Na^+ and Cl^- into myotubes over 30 min (given that net glutamine influx into myotubes under such conditions is maintained for several hours (Tadros *et al.* 1993*a*)) would increase solute concentration by 180 nosmol (mg cell protein)$^{-1}$, which is equivalent (given an initial 2·95 μl cell water (mg protein)$^{-1}$; Table 1) to a 61 mosmol l^{-1} increase in intracellular osmolarity. Assuming osmotic equilibration occurs, this potential increase in osmolarity would generate a 19% increase in cellular hydration. This calculated value represents a degree of cell swelling reported to affect transport and metabolism in the liver (Häussinger *et al.* 1994), but it is lower than the measured (35%) increase in myotube hydration under such circumstances (Table 1), possibly because the calculation underestimates the true glutamine plus Na^+ transport stoichiometry. Alternatively, it is possible that additional mechanisms for increasing intracellular osmolarity are recruited to effect the overall volume increase after the initial 'trigger' of swelling due to Na^+-coupled glutamine uptake. Such mechanisms may include those responsible for the cell swelling induced by insulin in the absence of glutamine. In hepatocytes (Häussinger *et al.* 1994), these appear to be Na^+–H^+ exchange and Na^+–K^+–Cl^- cotransport, which raise intracellular ion concentrations resulting in osmotic swelling.

The increased glutamine uptake observed during cell swelling is Na^+ dependent, Li^+ tolerant but not inhibited by MeAIB and involves increased transport V_{max}, characteristics which are all consistent with the conclusion that the increased transport capacity results from stimulation of system N^m (the insulin-stimulated glutamine transporter in skeletal muscle (Hundal *et al.* 1989; Tadros *et al.* 1993*a*)). It is also conceivable that additional pathways for glutamine movement across the sarcolemma (e.g. stretch-activated pores or channels) may be involved to a minor extent. It is, however, unlikely that the increase in glutamine uptake following hyposmotic exposure is due to movement through poorly selective anion channels because the channel blockers SITS, DIDS and NPPB do not prevent cell swelling-induced increases in glutamine uptake (Fig. 6). There is a good correlation between Na^+-dependent glutamine transport rate and myotube cell volume under the variety of experimental circumstances used in the present study, as summarized in Fig. 7. We suggest from this evidence that there may be a direct association between the activity of system N^m and myotube cell volume. Indeed, a very recent report (Schliess, Schrieber & Häussinger, 1995) has shown rapid (~1 min) activation of mitogen-activated protein kinases (extracellular signal-regulated protein kinase-1 and -2) by hyposmotic swelling of H4IIE hepatoma cells; similar activation of kinase signalling pathways in myotubes may be associated with the activation of system N^m.

The effects of volume changes on myotubes will include changes in intracellular concentrations of amino acids, Na^+, ATP and, possibly, significant changes in membrane potential, any or all of which may have a generalized influence on the activity of Na^+-dependent amino acid transporters. Such influences cannot account for the observed effect on glutamine transport, which appears to

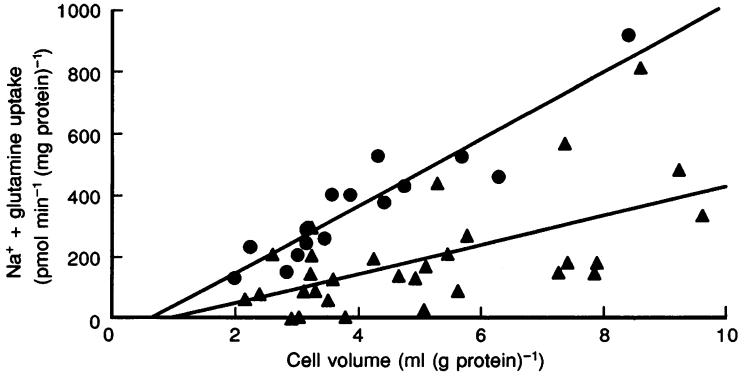

Figure 7. The relationship between cell water space and Na^+-dependent glutamine uptake in myotubes under a variety of experimental conditions

The values shown are Na^+-dependent glutamine uptakes (0·05 mM) estimated from total glutamine uptakes (measured in NaCl-containing medium) by subtraction of a mean glutamine uptake value in ChoCl medium (90 pmol min^{-1} (mg protein)$^{-1}$); a value largely unaffected by 30 min exposure to insulin, amino acid or anisosmotic medium; see Fig. 3). These values were estimated from data shown in Table 1 (121 mM NaCl values, ●) and Fig. 4 (60 mM NaCl values, ▲; using all the available time-points in which glutamine transport and water space were measured in the same myotube preparation). Significant ($P < 0·001$) positive correlation between glutamine transport and water space was observed at both 121 mM NaCl (correlation coefficient (r), 0·93; 15 d.f.) and 60 mM NaCl (r, 0·61; 29 d.f.). Regression lines were fitted by least-squares analysis; slopes were significantly different from zero ($P < 0·001$) in both cases.

be relatively specific, because myotube uptake of glutamate (which also has a major Na^+-dependent component (Low *et al.* 1994)) is unaffected by short-term exposure to hyposmotic medium and uptake of MeAIB (a substrate of Na^+-dependent system A) actually decreases in swollen cells. This decrease in system A activity is consistent with its reported role in cell volume regulation (Soler, Felipe, Casado, McGivan & Pastor-Anglada, 1993) and indeed, the shape of the cell hydration curve after hyposmotic exposure (Fig. 4*B*) is suggestive of some regulatory volume decrease over 30 min by mechanisms similar to those employed by other mesodermal cells such as fibroblasts (Dall'Asta, Rossi, Bussolati & Gazzola, 1994). It is quite possible that the volume regulatory mechanisms of myotubes include selective amino acid efflux after cell swelling (conceivably through stretch-activated ion channels for certain amino acids (Roy, 1995), although apparently not for glutamine; see above) but a Na^+-dependent transporter such as system N^m is unlikely to contribute to this process.

We have shown previously that glutamine transport in skeletal muscle may undergo adaptive upregulation in response to glutamine deprivation via a protein synthesis dependent mechanism ($t_{½}$ ~4 h) (Tadros *et al.* 1993*b*; Low *et al.* 1994), but the rapid (< 2 min) changes in glutamine transport observed following short term hyposmotic exposure makes it unlikely that a similar regulatory mechanism involving *de novo* protein synthesis is involved in this situation. The available evidence makes it more likely that the observed increase in transporter activity involves some mechanism such as direct activation of transporter proteins in the sarcolemma or the rapid recruitment of transport proteins from an intracellular pool, although a cycloheximide-sensitive component of insulin-stimulated glutamine transport in myotubes is observed over longer (0·5–1 h) time periods (Tadros *et al.* 1993*a*). The microtubule system is suggested (Soler *et al.* 1993) to play a role in mediating the regulatory response of system A amino acid transport to changes in cell volume and it is feasible that such a mechanism could be involved in our present study. Longer-term regulation of amino acid transport activity in response to changes in cell volume may occur by mechanisms involving protein synthesis (Soler *et al.* 1993).

Glutamine is an important osmotic effector in many cell types (including hepatocytes, astrocytes and myotubes) and in mature human skeletal muscle (Abumrad *et al.* 1989; Christensen, 1990) but the activation of sarcolemmal glutamine transport associated with cell swelling appears unlikely to be part of the cell regulatory response to volume increase. Indeed, provision of glutamine in the medium tends to *increase* cell volume and this enhances the cell swelling produced by insulin; furthermore, net glutamine efflux from incubated rat skeletal muscle decreases after cell swelling (Parry-Billings *et al.* 1991).

Hepatic system N (a transporter with functional similarities to system N^m) is also activated in response to swelling of liver cells (Häussinger *et al.* 1990; Bode & Kilberg, 1991). The apparent shift towards increased net glutamine uptake in swollen cells has been hypothesized to represent a positive feedback mechanism acting to increase the gain of the coupling between the plasma concentration of glutamine and the intracellular metabolic control systems (Häussinger *et al.* 1990; Bode & Kilberg, 1991). Certainly the rate of Na^+-dependent glutamine uptake (and consequent amplification of myotubular swelling) is positively related to the prevailing external glutamine concentration. Plasma glutamine concentration is positively related to whole-body nitrogen balance (Abumrad *et al.* 1989; Christensen, 1990; Rennie *et al.* 1990) and the ability to sense the state of the general amino acid economy (from the extent of glutamine-induced cell swelling) might, for example, enable more sensitive and appropriate control of the intramuscular responses of protein and amino acid metabolism to insulin.

The present results are consistent with our hypothesis that stimulation of the Na^+-dependent glutamine transport activity of system N^m by insulin and increased cell volume is an integral component of the skeletal muscle anabolic signal. Our previous work has shown that system N^m exhibits pathophysiological modulation such that glutamine uptake is depressed and net release elevated during disease and injury (Rennie *et al.* 1986, 1989; Feng, Shiber & Max, 1990). These changes in transport characteristics and the resultant loss of control of glutamine uptake could diminish the anabolic role of glutamine, thus contributing to lean-tissue catabolism. An understanding of the mechanisms involved in the modulation of muscle glutamine transport could be used as a basis to develop pharmacological interventions designed to stimulate muscle glutamine uptake and muscle anabolism during disease and injury.

Abumrad, N. N., Williams, P., Frexes-Steed, M., Geer, R., Flakoll, P., Cersosmio, E., Brown, L. L., Melki, I., Bulus, N., Hourani, H., Hubbard, M. & Ghishan, F. (1989). Inter-organ metabolism of amino acids *in vivo*. *Diabetes Metabolism Review* **5**, 213–216.

Ahmed, A., Taylor, P. M. & Rennie, M. J. (1990). Characteristics of glutamine transport in sarcolemmal vesicles from rat skeletal muscle. *American Journal of Physiology* **259**, E284–291.

Barua, J. M., Wilson, E., Downie, S., Weryk, B., Cuschieri, A. & Rennie, M. J. (1992). The effect of alanyl-glutamine peptide supplementation on muscle protein synthesis in post-surgical patients receiving glutamine free amino acids intravenously. *Proceedings of the Nutrition Society* **51**,115*P*.

Bode, B. P. & Kilberg, M. S. (1991). Amino acid-dependent increase in hepatic system N activity is linked to cell-swelling. *Journal of Biological Chemistry* **266**, 7376–7381.

Christensen, H. N. (1990). Role of amino acid transport and counter-transport in nutrition and metabolism. *Physiological Reviews* **70**, 43–77.

Dall'Asta, V., Rossi, P. A., Bussolati, O. & Gazzola, G. C. (1994). Regulatory volume decrease of cultured human fibroblasts involves changes in intracellular amino-acid pool. *Biochimica et Biophysica Acta* **1220**, 139–145.

Feng, B., Shiber, S. K. & Max, S. R. (1990). Glutamine regulates glutamine synthetase expression in skeletal muscle cells in culture. *Journal of Cellular Physiology* **145**, 376–380.

Häussinger, D., Lang, F., Bauers, K. & Gerok, W. (1990). Interactions between glutamine metabolism and cell volume regulation in perfused rat liver. *European Journal of Biochemistry* **188**, 689–695.

Häussinger, D., Lang, F. & Gerok, W. (1994). Regulation of cell function by the cellular hydration state. *American Journal of Physiology* **267**, E343–355.

Häussinger, D., Roth, E., Lang, F. & Gerok, W. (1993). Cellular hydration state: an important determinant of protein catabolism in health and disease. *Lancet* **341**, 1330–1332.

Hundal, H. S., Rennie, M. J. & Watt, P. W. (1987). Characteristics of L-glutamine transport in perfused rat skeletal muscle. *Journal of Physiology* **393**, 283–305.

Hundal, H. S., Rennie, M. J. & Watt, P. W. (1989). Characteristics of acidic, basic and neutral amino acid transport in the perfused rat hindlimb. *Journal of Physiology* **408**, 93–114.

Low, S. Y., Rennie, M. J. & Taylor, P. M. (1994). Sodium-dependent glutamate transport in cultured rat myotubes increases after glutamine deprivation. *FASEB Journal* **8**, 127–131.

MacLennan, P. A., Brown, R. A. & Rennie, M. J. (1987). A positive relationship between protein synthetic rate and intracellular glutamine concentration in perfused rat skeletal muscle. *FEBS Letters* **215**, 187–191.

MacLennan, P. A., Smith, K., Weryk, B., Watt, P. W. & Rennie, M. J. (1988). Inhibition of protein breakdown by glutamine in perfused rat skeletal muscle. *FEBS Letters* **237**, 133–136.

Parry-Billings, M., Bevan, S. J., Opara, E. & Newsholme, E. A. (1991). Effects of changes in cell volume on the rates of glutamine and alanine release from rat skeletal muscle *in vivo*. *Biochemical Journal* **276**, 559–561.

Rennie, M. J, Ahmed, A., Low, S. Y., Hundal, H. S., Watt, P. W., MacLennan, P. A., Egan, C. J. & Taylor, P. M. (1990). Transport of amino acids in muscle, gut and liver: relevance of metabolic control. *Biochemical Society Transactions* **18**, 1140–1142.

Rennie, M. J., Hundal, H. S., Babij, P., MacLennan, P. A., Taylor, P. M., Watt, P. W., Jepson, M. M. & Millward, D. J. (1986). Characteristics of a glutamine carrier in skeletal muscle have important consequences for nitrogen loss in injury, infection and chronic disease. *Lancet* **ii**, 1008–1012.

Rennie, M. J., MacLennan, P. A., Hundal, H. S., Weryk, B., Smith, K., Taylor, P. M., Egan, C. J. & Watt, P. W. (1989). Skeletal muscle glutamine transport, intramuscular glutamine concentration and muscle protein turnover. *Metabolism* **38**, 47–51.

Rennie, M. J. & Scislowski, P. W. D. (1989). The relationship between glutamine and protein turnover in mammalian tissues. In *Nutrition in Clinical Practice*, ed. Hartig, W., Dieze, G., Weiner, R. & Fürst, P., pp. 33–45. Karger, Basel.

Roy, G. (1995). Amino acid current through anion channels in cultured human glial cells. *Journal of Membrane Biology* **147**, 33–44.

Schliess, F., Schrieber, R. & Häussinger, D. (1995). Activation of extracellular signal-regulated kinases Erk-1 and Erk-2 by cell swelling in H4IIE hepatoma cells. *Biochemical Journal* **309**, 13–17.

Scislowski, P. W. D., Niblock, A., Lindsay, Y., Weryk, B., Watt, P. W. & Rennie, M. J. (1989). Glutamine stimulates glycogen synthesis in skeletal muscle. *Clinical Nutrition* **8**, 97.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. T., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76–85.

Soler, C., Felipe, A., Casado, J., McGivan, J. D. & Pastor-Anglada, M. (1993). Hyperosmolarity leads to an increase in derepressed system A activity in the renal epithelial cell line NBL-1. *Biochemical Journal* **289**, 653–658.

Stehle, P., Zander, J., Albers, S., Puchstein C. H., Lawin, P. & Fürst, P. (1989). Effect of parenteral glutamine peptide supplements on muscle glutamine loss and nitrogen balance after major surgery. *Lancet* **i**, 231–233.

Tadros, L. B., Taylor, P. M. & Rennie, M. J. (1993*a*). Characteristics of glutamine transport in primary tissue culture of rat skeletal muscle. *American Journal of Physiology* **265**, E135–144.

Tadros, L. B., Willhoft, N. M., Taylor, P. M. & Rennie, M. J. (1993*b*). The effects of glutamine deprivation on glutamine transport and synthesis in primary tissue culture of rat skeletal muscle. *American Journal of Physiology* **265**, E935–942.

Varnier, M., Leese, G. P. & Rennie, M. J. (1995). Stimulatory effect of glutamine on glycogen accumulation in human skeletal muscle. *American Journal of Physiology* **269**, E309–315.

Vom Dahl, S., Hallbrucker, C., Lang, F. & Häussinger, D. (1991). Regulation of cell volume in the perfused rat liver by hormones. *Biochemical Journal* **280**, 105–109.

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